

Flow Cytometry

Richard R. Jahan-Tigh^{1,2}, Caitriona Ryan^{3,4,5}, Gerlinde Obermoser⁵ and Kathryn Schwarzenberger⁶

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Of the techniques available to both the clinician and the researcher, few are as widely employed as flow cytometry. With applications that range from common clinical laboratory tests, such as complete blood count with differential and monitoring of CD4 cell count in HIV patients, to advanced, multicolor flow cytometry used to identify subtypes of inflammatory cells active in diseases such as psoriasis and lupus, flow cytometry has become rapid, flexible, and sensitive.

THE FLOW CYTOMETRY PROCESS

Flow cytometry measures single cells “flowing” through a detector system. The process begins with the selection of fluorescent-labeled antibodies specific to cell-surface markers used to characterize the cell population of interest. These cell surface markers are usually glycoproteins called cluster of differentiation (CD) markers, and they help differentiate cell subpopulations (e.g., CD3⁺CD8⁺ for cytotoxic T cells). Flow cytometry can be performed on a variety of tissues, including peripheral blood, bone marrow aspirates, skin biopsies, and tissue culture cell lines (Macey, 2007). The sample is processed, for example, with enzymatic degradation, centrifugation, and/or filtration to isolate the cells of interest, and the resulting cellular suspension is “stained” with fluorescent antibodies. The single cell suspension is then introduced into the flow cytometer into a cell-free buffer solution called the sheath fluid, which flows toward a laser aimed at the solution’s path. Because the flow of the liquid through the tubing is laminar, or sheet-like, and the diameter of the tubing narrows along its path, the cells are forced to line up single file as they approach the laser (Figure 1). The fluorescent chemical bound to the antibody, called a fluorophore, is chosen based on the specific wavelength of laser present in each flow cytometer. If cells have the selected marker on the surface, the bound antibody–fluorophore will absorb the laser energy and subsequently release it in the form of a specific wavelength of light as the cells pass through the laser. The emitted light is detected by an opti-

WHAT FLOW CYTOMETRY DOES

- Flow cytometry is a sensitive, powerful method for simultaneously obtaining information on various cellular processes, including expression of surface markers, intracellular cytokine and signaling proteins, or cell cycle.
- Flow cytometry measures these characteristics on each cell individually in a high-throughput fashion and excels in characterizing heterogeneous cell populations.
- Flow cytometry is capable of sorting cells based on almost any of the features it measures.

LIMITATIONS

- Flow cytometry is limited by its requirement that analyzed cells be in suspension, making information on tissue architecture and cell–cell interactions unavailable.
- Cell subpopulations with similar marker expression are difficult to differentiate and analyses that employ more fluorophores are subject to signal spillover.
- Flow cytometry may generate massive amounts of data, making analyses complicated.

cal system that is sensitive to various wavelengths, allowing for information on multiple surface markers to be read simultaneously and collected by an adjoined computer. Specialized software then can graphically represent the distribution of the labeled cell populations in one-, two-, or three-dimensional formats (Figure 2).

A variety of fluorochromes are available that individually emit light of specific, different wavelengths while absorbing light of the same wavelength. This is the basis

¹Department of Dermatology, University of Texas Houston Medical School, Houston, Texas, USA; ²Department of Dermatology, University of Texas MD Anderson Cancer Center, Houston, Texas, USA; ³Department of Dermatology, Baylor University Medical Center, Dallas, Texas, USA; ⁴Mentor Dermatology Research Institute, Dallas, Texas, USA; ⁵Baylor Institute for Immunology Research, Dallas, Texas, USA and ⁶Division of Dermatology, University of Vermont College of Medicine, Burlington, Vermont, USA

Correspondence: Richard R. Jahan-Tigh, University of Texas Health Science Center at Houston, MD Anderson Cancer Center, 6655 Travis Street, Suite 980, Houston, Texas 77030, USA. E-mail: Richard.R.Jahantigh@uth.tmc.edu; Gerlinde Obermoser, Baylor Institute for Immunology Research, Flow Cytometry Shared Resource Laboratory, 3434 Live Oak, Dallas, Texas 75204, USA. E-mail: E64349@BaylorHealth.edu

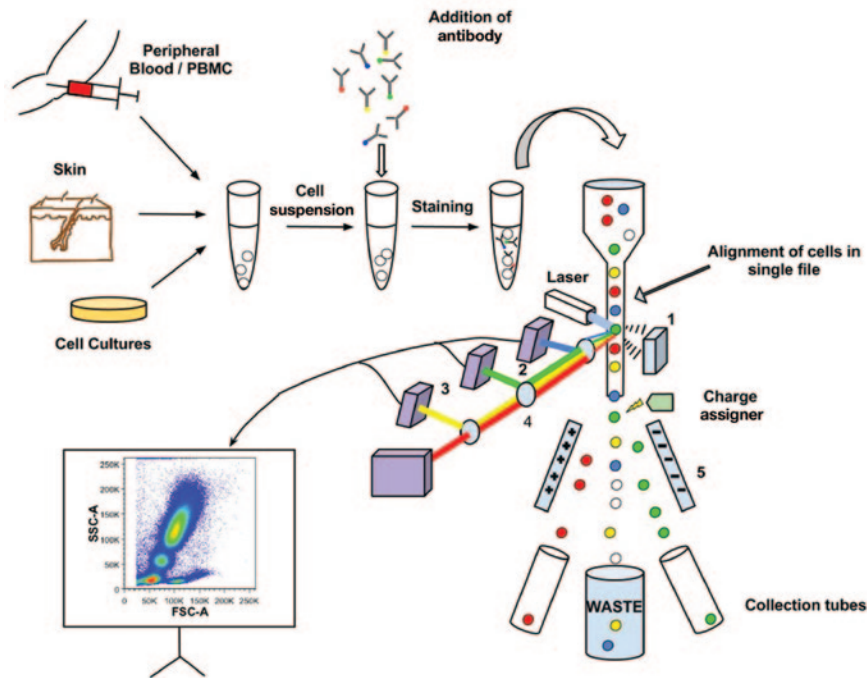


Figure 1. Schematic representation of a flow cytometer. For details please see text. (1) Forward-scatter detector, (2) side-scatter detector, (3) fluorescence detector, (4) filters and mirrors, and (5) charged deflection plates.

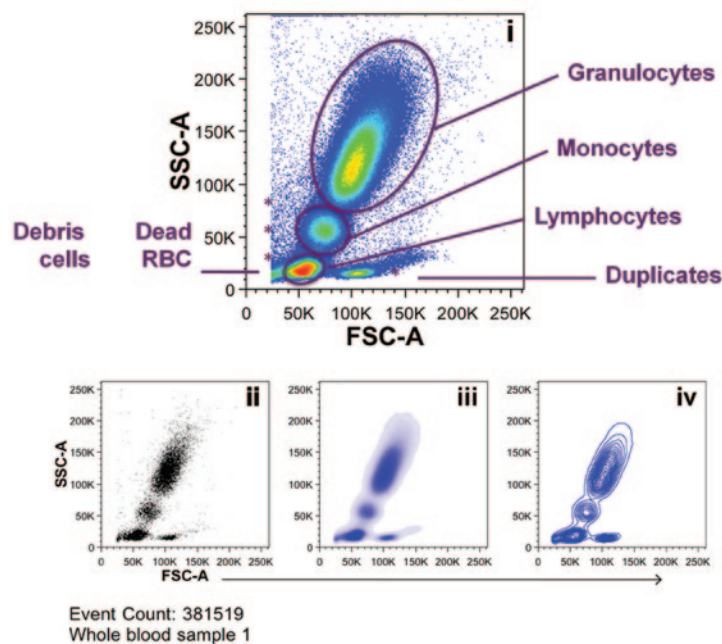


Figure 2. Scatter plots displaying the FSC (X-axis) and SSC (Y-axis) of lysed whole peripheral blood. The FSC scatter data provide information on the relative size of the cells, whereas the SSC data estimate the granularity. Four ways of displaying identical data: (i) pseudocolor dot plot (allows simultaneous information of rare events (dots) and high-frequency areas with dots of different color), (ii) dot plot where each dot represents one event (note that here only 10,000 events are shown to avoid oversaturation of dots), (iii) 5% probability density plot, and (iv) 5% probability contour plot, where the density of a population is translated in varying shades of color or concentric rings of varying distance. Major leukocyte populations as defined by FSC and SSC properties are shown in (i). FSC-A, forward scatter area; RBC, red blood cells; SSC-A, side scatter area.

for polychromatic flow cytometry, and it allows for a flow cytometry sample labeled with different fluorochrome–antibody complexes to be read simultaneously with one pass through the laser. Because emission spectra of fluorochromes overlap to varying degrees, a single detector may see fluorescence originating from more than one fluorochrome; this spillover must be removed using a mathematical algorithm, so that one detector reports signal from only one fluorochrome. This process is called “compensation.”

In addition to antibodies, fluorescent dyes are available that label the plasma membrane, DNA, or, for example, substrates for enzymes (e.g., apoptosis-inducing caspases or autophagy-related enzyme LC3). Thus, it is possible to simultaneously measure multiple parameters in a single cell by detecting cell-surface molecules defining the lineage (CD3⁺CD4⁺ T-cell lymphocytes), activation status (HLA-DR, CD38), phosphorylation status of signal transduction molecules (“phospho-flow”), intracellular cytokine production, cell cycle phase (via DNA content), viability, calcium flux, oxidative burst, etc.

FLOW CYTOMETRIC CELL SORTING, “FLUORESCENCE-ACTIVATED CELL SORTING”

Cells of interest can be separated to very high purity (very often 99%) by cell-sorting flow cytometers. After cells are “interrogated” by laser light in the flow chamber, the single cell stream is broken accurately into tiny droplets by a fine nozzle vibrating at ultrasonic frequency. Very rapid computation of the signals elicited by the cell in the flow chamber makes it possible to deflect droplets carrying cells of interest using positive, neutral, or negative electric charges (Figure 1). The droplets enter an electromagnetic field and are pushed, based on their charge, into different sorting containers.

FLOW CYTOMETRY DATA ANALYSIS

In addition to the fluorescence data, optical information known as forward scatter (FSC) and side scatter (SSC) is obtained based on the angle of light emitted from the analyzed cell. Classically, FSC is light scattered at a small angle and detected by a sensor on the opposite side of the 488-nm/blue laser source (Figure 1). FSC essentially provides information correlating with cell size. Light that scatters off the cell at a 90° angle, called “side-scatter,” is picked up by an adjacent series of sensors and provides information on the granularity of the cell. This could be useful, for example, when trying to distinguish between lymphocytes and granulocytes. The scatter plot is one of the most basic means of visualizing flow cytometric data, and the information is plotted with the X-axis as FSC intensity and the Y-axis as SSC intensity (Figure 2). The axes of the plots are labeled with the name of the fluorochrome and the surface marker (e.g., FITC–CD3 for fluorescein isothiocyanate-labeled CD3 cells). Cells positive for both markers will be located in the upper right quadrant, whereas cells negative for both will be located in the bottom left quadrant (Figure 3). For the scatter dot plots the axes are linear, whereas in fluorescence plots, because of the wide range of fluorescence signals, they are usually displayed logarithmically (Herzenberg *et al.*, 2006).

“Gating” is the term used to describe the selection of a subpopulation of cells for analysis. For example, if further characterization of only lymphocytes within the white blood cells is desired, the region of the lymphocytes on the FSC–SSC plot would be outlined and a gate placed on the lymphocyte cell population. How this is then represented is described in the following example: Jones *et al.* (2012) explored the expression patterns of T-plastin (PLS3) on Sézary cells. Single-color flow analysis was first used to distinguish the PLS3⁺ cells from the PLS3[−] cells, with the cell marker being measured on the X-axis and the number of counts on the Y-axis (Figure 4,

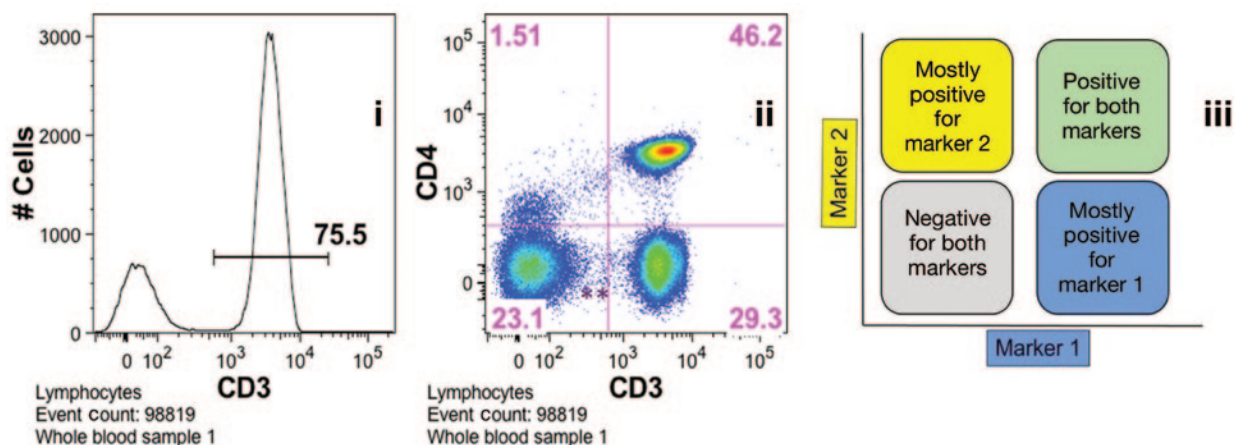


Figure 3. Gating strategy to define lymphocyte subsets. (i) Histogram (univariate) plot of CD3 expression; this one-dimensional graph corresponds to a typical bar chart and is called “histogram” in flow cytometry. (ii) Pseudocolor plot of CD3 versus CD4. This display gives a better view on the distribution of CD3 expression than the histogram, in particular for **CD3 low expressing cells; note both axes are logarithmic, unlike the linear axes of scatter plots in Figure 2. (iii) Cartoon detailing interpretation of quadrant gates of (ii). CD3⁺CD4⁺ cells are displayed in the top right quadrant (46.2% of lymphocytes). CD, cluster of differentiation.

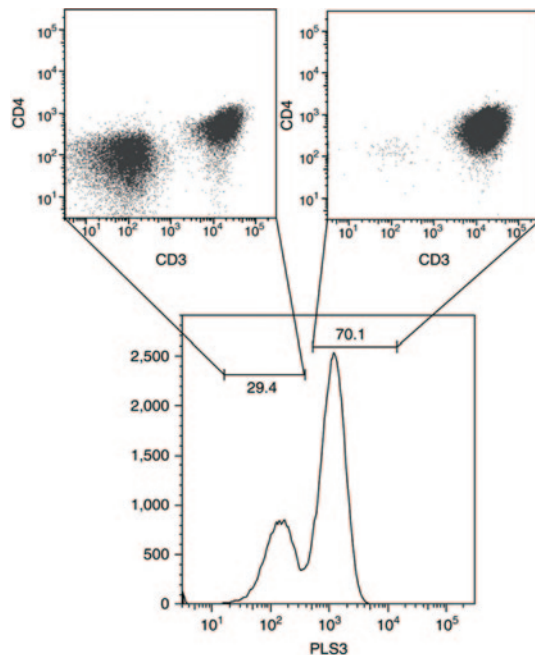


Figure 4. An example of gating in which the single-color flow analysis is shown with the X-axis labeled with the name of the fluorescently labeled cell marker, PLS3, on a logarithmic scale and the number of counts of the marker on the Y-axis. Two cell populations are seen: one that is positive for PLS3 (on the right) and a second that is negative (on the left). The fluorescent by fluorescent dot plot on the upper left is “gated on”—or restricted to—those cells that were PLS3 negative and demonstrates a large group of heterogeneous cells with weak staining for CD3 and CD4 and a separate population with stronger CD3/CD4 staining. In the upper right, PLS3⁺ cells are gated on and the CD3 by CD4 fluorescent plot shows that most of the analyzed cells in that gate are CD3/CD4, although the CD3 is an order of magnitude higher than CD4. Taken together, this experiment demonstrated that the Sézary cells from this patient were mostly, but not all, PLS3⁺ (Jones *et al.*, 2012). CD, cluster of differentiation; PLS3, T-plastin.

bottom). They then “gated on,” or only allowed analysis of, the PLS3⁺ cells (upper left) and then gated on the PLS3⁺ cells (upper right) and plotted both populations by CD3 by CD4 fluorescence. This demonstrated that a diverse population existed within the PLS3⁺ cells, most of which stained weakly for CD3 and CD4, but that there was also a separate population that stained more strongly for CD3 and CD4, representing CD3⁺CD4⁺PLS3⁺ Sézary cells. When only PLS3⁺ cells were examined (i.e., the CD3, CD4 plot gating on PLS3⁺ cells), most of them were CD3⁺CD4⁺, leading the authors to conclude that although PLS3⁺ may label a substantial subset of Sézary cells, some tumor cells will be missed if relying only on PLS3 staining. Figure 4 demonstrates another common convention in flow cytometry, which is either to show the percentage of a certain cell population with a line and a number, as in this figure, or to split the plots into quadrants or geometric shapes with an adjacent number representing the percentage of the total cell population delineated.

WHAT ARE THE MAJOR ADVANTAGES AND DISADVANTAGES OF FLOW CYTOMETRY, AND HOW DOES IT COMPARE WITH OTHER TECHNIQUES?

Flow cytometry is the most important technology for generating correlative information about single cells within heterogeneous sample preparations and in a high-throughput fashion. Advances in technology and fluorophore chemistry allow for the rapid and quantitative measurement of up to 20 parameters of cell phenotype simultaneously in a highly sensitive and reproducible manner (De Rosa *et al.*, 2001). The greatest advantage of polychromatic flow cytometry is its high specificity for discrete cell subsets and rare populations, as demonstrated by Kagami *et al.* in their pivotal psoriasis study (Kagami *et al.*, 2010; see Figure 5). Rare cell subsets with frequencies as low as 0.01% (e.g., antigen-specific cytokine-producing cells) can be detected. In addition, flow cytometric cell sorting allows for isolation of cells of interest to very high purity.

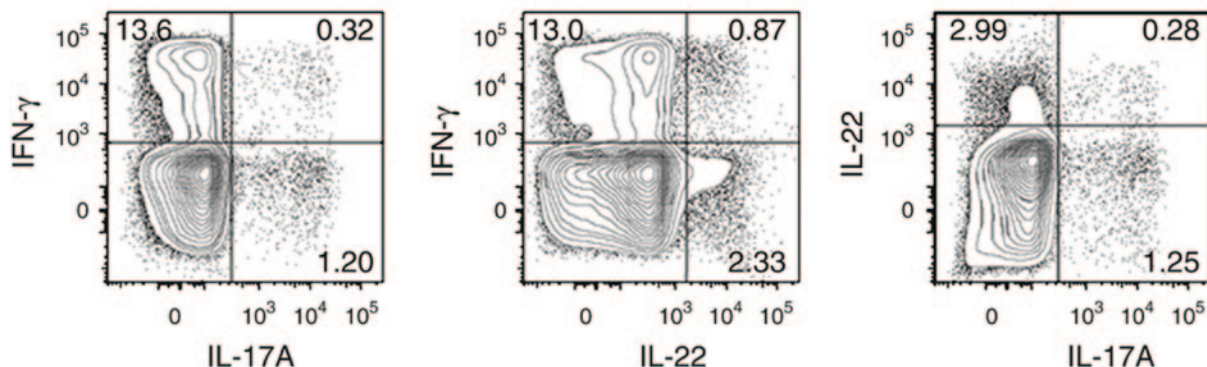


Figure 5. Kagami *et al.* (2010) reported an increased frequency of Th17, Th22, and Th1 cells, specialized CD4⁺ Th lymphocytes producing IL-17A, IL-22, and IFN- γ , respectively, in untreated psoriasis patients compared with healthy controls using seven-color flow cytometry. The fluorescence plots are gated on CD4⁺ lymphocytes that have been stimulated for 6 hours with phorbol myristate acetate, followed by staining for IL-17A, interferon (IFN)- γ , and IL-22. Of note, most cells producing IL-22 do not produce IL-17A or IFN- γ at the same time: in the middle plot, a total of 3.2% of cells are positive for IL-22 staining (2.33% cells produce only IL-22 and not IFN- γ , whereas 0.87% produce both cytokines). Similarly, in the right fluorescence plot, a total of 3.27% of cells produce IL-22 (2.99% of cells are single positive for IL-22 and 1.25% are double positive for IL-22 and IL-17A). A similar observation can be made for IL-17A-producing cells. CD, cluster of differentiation; IL, interleukin; Th, T helper.

Highly multiplex data can also be generated by genome-wide transcriptional profiling (microarray, RNAseq); however, these data do not allow for the distinction of signals on a single-cell basis. Likewise, various immunoblotting techniques (such as western blot) and polymerase chain reaction use cellular lysates and do not provide information about single cells. Enzyme-linked immunosorbent assays measure soluble, and not cell-bound, markers.

WHAT ARE THE MAJOR LIMITATIONS OF THIS TECHNOLOGY?

Cells must be in a single-cell suspension to be evaluated in a flow cytometer. Although this is not a problem for blood cells, tissues must be disrupted, which can affect cellular function. Also, information about tissue architecture is not available. The number of parameters per cell that can be measured simultaneously is limited by the number of detectors to less than two dozen, and, because of the spillover effect, only 6–12 color experiments are performed routinely in most laboratories (Roederer, 2001). Validation of results is required using the simultaneous detection of multiple markers to increase specificity or the analysis of stimulated cytokine production of isolated cells to further confirm their phenotype. An important limitation to flow cytometry is a surprising lack of standardization in assay and instrument set-up; standards are also lacking in how flow data are analyzed and reported, although numerous efforts have been launched recently (Maecker *et al.*, 2010; Britten *et al.*, 2011). Finally, because of the massive amount of data generated, flow data analysis can become very complicated and relies almost exclusively on gating by a human expert. Automated multidimensional visualization and gating tools as well as postanalysis data aggregation models for flow cytometry data (Qiu *et al.*, 2011) are being developed to fill this gap.

SUMMARY AND FUTURE DIRECTIONS

New developments aim to combine the advantages of flow cytometry (i.e., measuring multiple parameters on a single-cell level) with transcriptional profiling or microscopy (imaging flow cytometry) or to push into the realm of “single-cell proteomics” (Irish *et al.*, 2006) by adding many more “detectors” with mass spectroscopy. Cytometry by time of flight, or mass cytometry, employs time-of-flight mass spectrometry to discriminate a set of more than 40 heavy metal ion-labeled antibodies without the drawbacks of fluorescence spillover or autofluorescence (Bendall *et al.*, 2011, 2012). The Fluidigm Biomark system allows the quantification of mRNA levels using highly multiplexed real-time polymerase chain reaction using special microfluidic devices, thereby allowing detection of up to more than 100 gene transcripts from a single cell (Bagwell, 2011).

Flow cytometry is a pivotal tool for the analysis of cell subsets and their complex interplay in immunological and biological processes. It has advanced our understanding of the immune system and is likely to play a fundamental role in the future in identifying biomarkers for disease prognosis or treatment response (“companion diagnostic tests”) and the development of individualized medicine.

QUESTIONS

1. Side scatter (SSC) and forward scatter (FSC) provide information on _____ and _____, respectively.
 - A. Tissue architecture, granularity
 - B. Granularity, size
 - C. Size, cell–cell interactions
 - D. Cell-surface markers, intracellular signaling
2. “Gating” refers to:
 - A. The process of cells lining up single file before entering the laser path.
 - B. The field the cells enter during the sorting process.
 - C. The restriction of a portion of the analyzed cells for further analysis.
 - D. The overlapping fluorophore signals generated in flow experiments with many fluorophores.
3. In a fluorescent by fluorescent scatter plot, cells present in the upper right quadrant of the plot are generally:
 - A. Negative for one marker, positive for the other.
 - B. Negative for both markers.
 - C. Positive for aberrant marker expression.
 - D. Positive for both markers.

Answers to the questions and an opportunity to comment on the article are available on our blog: http://blogs.nature.com/jid_jottings/.

CONFLICT OF INTEREST

Caitriona Ryan has acted as a speaker for Jansen-Cilag and Pfizer and as an advisory board member for Galderma and has received grant support from Abbott.

SUPPLEMENTARY MATERIAL

Answers and a PowerPoint slide presentation appropriate for journal club or other teaching exercises are available at <http://dx.doi.org/10.1038/jid.2012.282>.

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